Regulation of prefrontal glutamate by the endogenous neuromodulator kynurenic acid as measured by rapid electrochemistry: relevance to schizophrenia

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ABSTRACT

Schizophrenia is one of the most debilitating neuropsychiatric disorders, with a prevalence of approximately 1% worldwide. Symptoms include impairing deficits in cognitive processes (e.g., working memory, attention, and cognitive flexibility) which are currently elusive to treatment. Alpha-7 nicotinic acetylcholine receptors (α7 nAChRs) may be a critical link between the dysregulated cortical glutamatergic transmission and cognitive deficits in schizophrenia. Elevated levels of the endogenous, astrocyte-derived neuromodulator kynurenic acid (KYNA), a potent, non-competitive α7 nAChR antagonist, in the brains and cerebrospinal fluid (CSF) of schizophrenic patients suggests that KYNA binding may cause or exacerbate cortical dysregulation in schizophrenia. Thus, we used a state-of-the-art microelectrode array (MEA) to amperometrically examine the regulation of glutamate release by KYNA in the prefrontal cortex (PFC) of intact, freely moving rats. The MEA allows for the selective quantification of glutamate levels with second-by-second resolution (500-800 ms), a limit of detection of ≤0.5 μM, and outstanding spatial resolution (<100 μm). Intraperitoneal (i.p.) injections of L-kynurenine (25 and 50 mg/kg), KYNA's bioprecursor, dose-dependently decreased basal glutamate levels in PFC (nadir after 50 mg/kg kynurenine: 31% decrease from baseline values). However, co-administration of galantamine (3 mg/kg), a drug that competes with KYNA at an allosteric potentiating site of the α7 nAChR, attenuated kynurenine-mediated reductions in cortical glutamate. These data in intact animals demonstrate KYNA's α7 nAChR-mediated regulation of glutamate release in the PFC. Drugs that normalize cortical glutamatergic function by attenuating KYNA production or by countering its effects on α7 nAChRs may be effective cognition-enhancing therapeutics in schizophrenic patients.

Note: The experiments presented in this thesis also appear in a manuscript (Konradsson-Geuken et al., 2010), which is in revisions for the journal *Neuroscience.*

INTRODUCTION

Schizophrenia is one of the most severe and debilitating neuropsychiatric disorders, with a prevalence of approximately 1% worldwide (Perala et al., 2007). The disease results from the interaction of multiple genetic and environmental factors, independent of geographic, cultural, or socio-economic variables (Harrison & Weinberger, 2005). The characteristic symptoms of schizophrenia fall into three categories: positive, negative, and cognitive symptoms (Andreasen, 1995). Positive symptoms reflect the distortion of normal thoughts in both form and content, and include hallucinations and delusions. Negative symptoms involve the decline or loss of normal behavior, and include affective flattening and social withdrawal. Cognitive symptoms result from impairments in executive function, and include deficits in working memory, attention, and cognitive flexibility.

While positive symptoms in most patients are reduced with first generation antipsychotic drugs (FGAs), these treatments have minimal effect ameliorating negative and cognitive symptoms, and may even exacerbate them (King, 1998). Treatment with FGAs is associated with a high incidence of extrapyramidal side-effects (EPS), such as acute dystonia and parkinsonism (Hansen et al., 1997). Treatment with second generation antipsychotic drugs (SGAs; e.g., clozapine) reduces positive symptoms, results in a lower incidence of EPS, and also has some effect reducing negative symptoms (Dayalu & Chou, 2008). However, SGAs may cause severe side effects which can lead to diabetes or cardiovascular disease (Kane et al., 1988). Despite significant advances in pharmacological treatment, nevertheless, schizophrenia remains prevalent and debilitating, largely due to the absence of treatments that address the disruptions in executive function. These impairments are integral because they accompany (Saykin et al., 1994) and may even predate the first psychotic episode (Jones et al., 1994). Additionally, there is a positive correlation between the severity of

cognitive dysfunction and the declining quality of life of schizophrenic patients (Green et al., 1996). The cognitive class of symptoms in schizophrenia is also the most predictive of social disintegration. Thus, the cognitive deficits of schizophrenia are the most devastating symptoms, but are also currently the most elusive in treatment (Keefe, 2007).

Recent clinical findings support the theory that dysregulated cortical glutamatergic transmission contributes to the pathophysiology of schizophrenia (Lewis & Moghaddam, 2006; Patil et al., 2007). The ability of phencyclidine, a glutamate N-methyl-D-aspartic acid receptor (NMDA-R) antagonist, to induce schizophrenia-like symptoms in healthy volunteers (Luby et. al, 1962) and to exacerbate the psychotic symptoms and cognitive deficits in schizophrenic patients (Javitt & Zukin, 1991) is the strongest evidence linking glutamatergic dysfunction to the pathophysiology of schizophrenia.

There is also evidence that altered cortical cholinergic function is a contributing factor in schizophrenia (Hyde and Crook, 2001; Sarter et al., 2005). Post-mortem studies have reported a decrease in muscarinic (Crook et al., 2001) and nicotinic (Breese et al., 2000) ACh receptor binding in the prefrontal cortex of schizophrenic patients. The focus on the alpha7 nicotinic ACh receptor (α7 nAChR) is based upon demonstrations of a) impaired nicotinic ACh transmission in schizophrenia (Leonard & Freedman, 2006), b) reduced expression of the α7 nAChR protein in the prefrontal cortex (PFC) of schizophrenic patients (Guan et al., 1999), c) an association between the α7 nAChR gene and disease transmission (Martin et al., 2007), and d) cognition-enhancing effects of nAChR agonists in schizophrenic patients (Olincy et al., 2006). In fact, there is a growing literature in animals (Levin & Rezvani, 2007) and humans (Harris et al., 2004) that stimulation of nAChRs (particularly the α7 subtype) is associated with the facilitation of several cognitive behaviors including working memory and attention.

Due to the localization of release-promoting α7 nAChR on cortical glutamatergic and cholinergic nerve terminals (Gotti et al., 2006; Lambe et al., 2003; Dickinson et al., 2008; Duffy et al., 2009) as well as the proximity of cholinergic and glutamatergic terminals in the PFC (Marchi et al., 2002; Rousseau et al., 2005; Wang et al., 2006; Konradsson-Geuken et al., 2009), it is likely that α7 nAChRs also mediate cortical glutamate release. Thus, impairments in cholinergic and glutamatergic functioning may be critical links between the dysregulated prefrontal circuitry underlying the cognitive deficits in individuals with schizophrenia (Sarter et al., 2005).

The relevance of glutamate and acetylcholine (ACh) hypofunction is also supported by elevated levels of kynurenic acid (KYNA), a potent non-competitive α7 nAChR antagonist (Hilmas et al., 2001) in the brain (Schwarcz et al., 2001) and cerebrospinal fluid (CSF; Erhardt et al., 2001) of post-mortem schizophrenic patients. KYNA is a metabolite of the kynurenine pathway of tryptophan degradation (Kiss et al., 2003; see Figure 1), and is formed by the transamination of L-kynurenine (kynurenine) catalyzed by several kynurenine aminotransferases (KAT; Turski et al., 1989), especially KAT II. A major locus of the enzyme KAT II is astrocytes (Guidetti et al., 2007), which subsequently release KYNA into the extracellular milieu (Turski et al., 1989; see Figure 2). At nanomolar concentrations, KYNA negatively modulates α7 nAChRs by binding to an allosteric site (Lopes et al., 2007). Recent studies investigating the bi-directional effects of fluctuating KYNA concentrations on dopamine, ACh, and glutamate in the PFC (Wu et al., 2006; Zmarowski et al., 2009; Wu et al., 2010) suggest that KYNA binding may cause or exacerbate dysregulation of the PFC in schizophrenia.

These experiments were designed to examine further the relationship between KYNA and glutamate in the PFC. **First**, we observed the effects of systemic administration of kynurenine, which elevates KYNA levels throughout the brain (Rassoulpour et al., 2006), on cortical glutamate levels in the PFC. As KYNA negatively

modulates the α7 nAChR by binding to its allosteric potentiating site (Lopes et al., 2007), we previously demonstrated that local infusion of KYNA tends to reduce cortical acetylcholine release (Zmarowski et al., 2009). Furthermore, due to the localization of release-promoting nAChRs on cortical glutamate terminals (Gotti et al., 2006), α7 nAChR ligands will also likely affect cortical glutamate release (Konradsson et al., 2009). If basal glutamate release is mediated by the α7 nAChR, **it is hypothesized** that systemic administration of kynurenine (25 mg/kg or 50 mg/kg), a bio-precursor to KYNA (see Figures 1 and 2), will dose-dependently decrease basal glutamate levels in PFC.

Next, additional animals were co-administered galantamine with kynurenine to test whether the effects of KYNA on glutamate levels were in fact mediated by α7 nAChRs. Galantamine, an allosteric potentiating ligand (APL) of the α7 nAChR, binds to a site similar to that bound by the negative modulator KYNA, but with opposite effects (Lopes et al., 2007). Pre-clinical studies indicate that, in low (micromolar) doses, galantamine acts as a potent positive allosteric modulator of the α7 nAChR (Samochocki et al., 2003), and enhances the antipsychotic effects of the typical antipsychotic raclopride (Wiker et al., 2008). The effect of galantamine on cognitive performance suggests the prospective benefit of allosteric potentiating ligands of α7 nAChR as adjunctive treatments for schizophrenia if administered at effective doses (Buchanan et al., 2008). Thus, if the reductions in cortical glutamate observed in Experiment 1 are in fact mediated by α7 nAChR, **it is hypothesized** that systemic administration of galantamine (3 mg/kg; 5 min pre- and 3 hr post-kynurenine injection) will *consistently* attenuate reductions in basal glutamate levels in PFC.

Extracellular glutamate levels were recorded in freely-moving animals with a glutamate-sensitive, amperometric microelectrode array (MEA) which wields unmatched temporal and spatial resolution.

METHODS

Animals

Male Wistar rats weighing 250–450 g were used as subjects in these experiments. Animals were maintained in a temperature- and humidity-controlled room on a 12:12-hour light:dark cycle (lights on at 06:00 a.m.) and individually housed in plastic cages lined with corn-cob bedding. Animals had access to food and water *ad libitum*. All procedures involving animals were approved by The Ohio State University Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals. As such, all efforts were made to minimize animal suffering and to reduce the number of animals used.

Materials

L-Kynurenine (sulfate form; 'kynurenine'), L-ascorbic acid (AA), dopamine (DA), L-glutamate (monosodium salt), glutaraldehyde [25% (w/v) in water], bovine serum albumin (BSA), and H_2O_2 were obtained from Sigma Aldrich Corp. (St. Louis, MO, USA). Galantamine was a research gift from Janssen Pharmaceutica (Beerse, Belgium). L-Glutamate oxidase (GluOx; EC 1.4.3.11) was purchased from Seikaghaku America, Inc. (East Falmouth, MA, USA). Meta-phenylenediamine (*m*-PD) was purchased from Acros Organics (Fairlawn, NJ, USA). All solutions were prepared using distilled, deionized water.

Coating of glutamate-sensitive microelectode array

The MEA used for these freely-moving experiments (Figure 3A) consists of a ceramic paddle containing, at its tip, four platinum (Pt) recording sites. The paddle of the MEA interfaces with a pre-amplifier headstage (see Rutherford et al., 2007 for more details on

this assemblage). Figure 3B is a magnified view of the tip of the MEA, revealing the four 15 x 333 μm Pt recording sites arranged in pairs beginning approximately 100 μm from the electrode tip. One pair of recording sites was designated to be sensitive to glutamate plus other endogenous electroactive species and was coated with glutamate GluOx (2%, 1 unit/1 μL, 100 nL), bovine serum albumin (BSA, 1%), and glutaraldehyde (0.125%). The remaining pair served as a sentinel site, sensitive to the oxidation of the same molecules except for glutamate. This coating arrangement and the design of the MEA allows for a self-referencing procedure (see below) in which the current derived *exclusively* from the oxidation of glutamate can be isolated (Day et al., 2006; Rutherford et al., 2007; Konradsson-Geuken et al., 2009). To control for the micro-environments into which a particular pair of sites could be implanted, the positioning of the glutamate-sensitive versus sentinel sites, relative to the tip of the MEA (i.e., more ventral vs. more dorsal pair) was counterbalanced within a treatment condition. Coated MEAs were allowed to dry for ≥ 2 days at room temperature (25°C) and low humidity prior to *in vitro* calibration (see below). *m*-PD (5.0 mM) was then electropolymerized onto all sites in order to reduce access of potential electroactive interferents, including AA and catecholamines, to the Pt recording sites (Mitchell, 2004). Electroplating was conducted in bubbled nitrogen using cyclic voltammetry (peak-to-peak amplitude of 0.25 V every 0.05 sec for 25 min).

Detection of glutamate-generated signals

The enzyme detection scheme for generating the current derived by the selective oxidation of glutamate is more fully elaborated elsewhere (Day et al., 2006; Rutherford et al., 2007; Konradsson-Geuken et al., 2009), but is described briefly in Figure 4 below. The various coatings of the Pt electrode surface for the two glutamate sensitive channels

are indicated by the shaded columns on the left and a schematic illustrating the two pairs of MEA recording sites appears in the middle. The shaded columns on the far right depict the events occurring at the electrode surface of the sentinel channels. In all channels, access to the Pt surface by DA is essentially blocked by *m*-PD. On the glutamate-sensitive channels (Figure 4, left), glutamate is oxidized by GluOx, generating α-ketoglutarate and H_2O_2. Because the MEA is maintained at a constant potential of +0.7 V, the H_2O_2 reporting molecule is oxidized, yielding two electrons. The resulting current is then amplified and recorded by a FAST-16 mkI recording system (Quanteon, LLC, Nicholasville, KY). On the sentinel channels, extracellular glutamate reaches the Pt surface but in the absence of GluOx no oxidation current is generated. Any current detected is due to endogenous electroactive molecules other than glutamate.

***In vitro* calibration of microelectrodes**

MEAs were calibrated prior to implantation using the FAST-16 mkI electrochemical recording system. Constant potential amperometry was conducted using an applied potential of +0.7 V versus a Ag/AgCl reference electrode. Calibrations were performed in a stirred solution of phosphate-buffered saline (PBS; 0.05 M, 40 mL, pH 7.4, 37°C). After stabilization, AA (250 μM), glutamate (3 x 20 μM), DA (2 μM), and H_2O_2 (8.8 μM) were sequentially added to the calibration beaker (see Figure 5). Amperometric signals were acquired at a rate of 2.0 Hz. The slope (sensitivity, nA/μM glutamate), limit of detection (L.O.D., μM glutamate), selectivity (ratio of glutamate over AA), and linearity (R^2) were calculated. In order to be used for subsequent *in vivo* recordings, the MEAs had to conform to the following calibration criteria (single electrode mode): a) similar background current (i.e., no greater than a 20 pA difference between the glutamate-sensitive and sentinel channels), b) linear response to increasing concentrations of

glutamate (R^2 > 0.998), c) a minimum slope of -3.0 pA/μM glutamate, d) an L.O.D. of ≤0.5 μM, and e) a high selectivity for glutamate over either AA or DA (i.e., >50:1).

Implantation of microelectrodes

Animals were anesthetized using isofluorane (2%, 0.6 L/min) and implanted with the microelectrode pedestal that contained the MEA and was connected to the miniaturized Rat Hat amplifier (Rutherford et al., 2007). MEAs were implanted unilaterally in the PFC, with coordinates determined from the atlas of Paxinos and Watson (1985) (AP: 2.7 mm anterior to bregma, ML: ±0.6 mm from midline, DV: 3.9 mm below dura; hemispheres counterbalanced within a treatment condition). The Ag/AgCl reference electrode was implanted in a contralateral site distant from the recording area.

***In vivo* recordings of systemic injections**

All electrochemical recordings in both Experiments 1 and 2 were conducted in freely moving rats in a large wooden box (57.2 cm H x 41.9 cm W x 17.0 cm L) 2-6 days after implantation of the MEA. Animals were placed in the recording box and connected to the head stage (see Figure 3C). Stable baseline signals were recorded following a 3-4 hr habituation period. In both experiments, the effects of systemic administration of test compounds on cortical glutamate were determined. In the first experiment, rats were treated with kynurenine (25 or 50 mg/kg, i.p.; n = 5/dose). At these doses, KYNA acts as a preferential α7 nAChR antagonist (Hilmas et al., 2001). While KYNA also blocks the $glycine_B$ site of the NMDA receptor (NMDA-R) with high potency in the absence glycine (see Figure 2), under physiological conditions KYNA is unlikely to inhibit NMDA-R (Hilmas et al., 2001). In the second experiment, the hypothesis that the effect of kynurenine was mediated by the antagonism of α7 nAChRs was tested. Thus, we

injected galantamine (3.0 mg/kg, i.p.), a positive allosteric modulator at low doses, that binds at a site of the α7 nAChR that is very similar to that targeted by KYNA (Lopes et al., 2007), 5 min prior to kynurenine (50 mg/kg, n = 5). Recordings continued for 6 hrs post-injection. Another group of rats (n = 5) was injected with both kynurenine and galantamine except that, in this case, the administration of galantamine was delayed until 3 hrs post-kynurenine.

Histology

At the conclusion of each experiment, animals were anesthetized with isoflourane and then given an overdose of pentobarbital. Brains were removed and stored in formalin (10%) for at least 24 hours, and then transferred to a sucrose solution (30%) for at least 3 days. Brains were sectioned using a cryostat; coronal (see Figure 6) and sagittal sections (50 μm) were mounted on gelatin-coated slides, stained using cresyl violet, and examined under a light microscope for verification of MEA placement in the PFC.

Data analyses

For the microelectrode experiments, group comparisons of absolute glutamate levels or various temporal dimensions of the glutamate signal were analyzed using one- and two-way analysis of variance. In some instances, when only percentage data were being compared, t-tests between independent means were utilized. Significance was defined as $P < 0.05$. All statistical tests were performed using SPSS for Windows (Version 17.0; Chicago, IL).

RESULTS

The following experiments utilized the glutamate-sensitive MEA to examine the effects of fluctuating KYNA levels on basal glutamate levels in the prefrontal cortex. Prior to implantation, MEAs were calibrated in a beaker with known concentrations of potential analytes. Figure 5 illustrates the results of a representative *in vitro* calibration. Current tracings following the administration of AA, glutamate, DA, and H_2O_2 (indicated by arrows) are shown from each of the 4 channels. The top two tracings (GluOx) depict current at the glutamate-sensitive channels, whereas the bottom two tracings (sentinel) illustrate current from the sentinel or background channels. The addition of AA to the beaker produced only minor increases in oxidation current, which were, importantly, comparable on all 4 channels. Serial additions of glutamate (20 μM aliquots) produced large, reproducible, and linear increases in current at the GluOx sites as the concentration of glutamate in the beaker was progressively elevated. In contrast, the signal on the sentinel channels did not change as a result of the addition of glutamate. Addition of DA produced only a slight change in current, indicating the effectiveness of the exclusion barrier *m*-PD, and this modest increase was comparable across the 4 channels. Finally, all 4 recording sites exhibited similar sensitivity to the reporter molecule, H_2O_2, which is a necessary condition for the self-referencing procedure used to isolate the current change caused by the oxidation of glutamate (Burmeister and Gerhardt, 2001).

Experiment 1a: Effects of systemic kynurenine on cortical glutamate levels

Figure 7 shows a representative MEA tracing following the systemic administration of kynurenine (50 mg/kg, i.p.). After the establishment of a stable baseline, kynurenine produced a gradual and protracted reduction in signal at the glutamate-sensitive site (top tracing, “GluOx”). There was only a minor negative drift in

the current output at the background sentinel site (second tracing, “Sentinel”). Self-referencing the sentinel from the GluOx sites yielded a signal that isolated current changes due only to the oxidation of glutamate (third tracing, “KYN self-ref”). The kynurenine-induced decrease in extracellular glutamate reached a maximum of -2.1 μM (-23%) from baseline by the end of the 6 hr recording session. Importantly, a control procedure conducted at the end of each session verified that the MEA had maintained its ability to record changes in extracellular glutamate levels. Thus, a local infusion of exogenous glutamate (0.25 mM) into the PFC at the nadir of the current output elicited a rapid and pronounced increase in signal. As prior studies reported that H_2O_2 is oxidized primarily at +0.7 V at the Pt recording sites versus Ag/AgCl, control recordings conducted at +0.25 V eliminated any differences between the GluOx and sentinel sites, verifying the glutamate signals *in vivo* (Pomerleau et al., 2003; data not shown). These observations were consistent with the interpretation that the self-referenced signal is generated by the oxidation of glutamate-derived H_2O_2 (Rutherford et al., 2007). The bottom tracing in Figure 7 (“Saline Self Ref”) illustrates a representative self-referenced record from an animal receiving a control injection of saline (0.9%, i.p.). This provides evidence that the injection *per se* did not lead to significant changes in current and that the signal was relatively stable over the entire recording session.

Table 1 summarizes group data on the magnitude and timing of the changes in the glutamate signal. Overall, kynurenine (50 mg/kg, n = 5) caused a significant decrease (-31%) compared to basal glutamate levels (t_4 = 4.67, P = 0.009). This reduction was evident within 6 min of the injection and reached a maximum 5 hrs later. This effect of kynurenine persisted throughout the 6 hr recording session.

Experiment 1b: Dose-dependency of kynurenine-mediated reductions in cortical glutamate levels

The ability of systemic kynurenine to decrease prefrontal glutamate levels was dose-dependent. Figure 8 illustrates the tracings from a representative animal treated with 25 mg/kg kynurenine (i.p.) (n = 5). While the baseline glutamate signal was similar to that seen when studying the effects of the higher dose, administration of the lower dose produced an effect that was smaller in magnitude (-1.3 μM, -12% from baseline) and of shorter duration (return to baseline within 4 hrs) than the reduction caused by the higher dose (cf. Figure 7). As summarized in the group data (Table 1), 25 mg/kg kynurenine caused a significant decrease (-11%) from basal glutamate levels (t_4 = 3.83, P = 0.019). Glutamate levels were reduced within 16 min after kynurenine and returned to basal values within 3 ½ hrs of the injection. 50 mg/kg kynurenine produced greater reductions in glutamate levels than 25 mg/kg ($F_{1,8}$ = 7.71, P = 0.02). While there was no significant dose-dependent difference in the onset of the effect, the time to reach maximal effect ($F_{1,9}$ = 44.70, P < 0.001) and effect duration ($F_{1,9}$ = 47.99, P < 0.001) were significantly longer for the higher dose than for the smaller dose. The dose- and time-dependent effects of systemically administered kynurenine on extracellular levels of KYNA and glutamate in the PFC are illustrated in Table 1.

Experiment 2: Tonic effects of co-administration of systemic galantamine and kynurenine on basal cortical glutamate levels

The kynurenine-induced reduction of prefrontal glutamate levels reflected the ability of increased KYNA levels to antagonize the α7 nAChR. Thus, pre-treatment with the positive α7 nAChR-modulator galantamine (3.0 mg/kg, i.p.) markedly attenuated the effects of 50 mg/kg kynurenine. Figure 9 illustrates a representative current tracing from the galantamine/kynurenine combination. The third trace from the top ("Gal/Kyn Self Ref") depicts the self-referenced glutamate signal following the combined treatment. Compared to the profile seen following 50 mg/kg kynurenine alone (Figure 7), the

combination of the two compounds produced a smaller maximal effect on glutamate (-1.3 μM), which reverted more quickly to basal levels (2 ½ hrs). The group data listed in Table 1 show that the combination of galantamine and kynurenine (n = 5) led to smaller decreases in glutamate ($F_{1,9}$ = 13.34, P = 0.006) and also shorter durations of the inhibition ($F_{1,9}$ = 255.36, P < 0.001) relative to kynurenine alone. In fact, pre-treatment with galantamine rendered the effects of 50 mg/kg kynurenine similar to the profile seen following the application of 25 mg/kg kynurenine alone. Treatment with galantamine, however, reduced the time to maximum effect relative to either dose of kynurenine ($F_{1,9}$ = 6.28, P = 0.04).

The bottom trace in Figure 9 ("Kyn-Gal Self Ref") illustrates a similar experiment, but the administration of galantamine was delayed until 3 hrs after the injection of kynurenine (50 mg/kg), when the inhibitory effect of kynurenine on glutamate was pronounced. Galantamine caused a clear reversal of the glutamate signal, which returned to baseline values within 90 min. These results demonstrated that the kynurenine-induced decrease in glutamate is *consistently* mediated by the α7 nAChR.

DISCUSSION

Previous studies suggest that the astrocyte-derived α7 nAChR antagonist KYNA, in endogenous (nanomolar) concentrations, indirectly modulates extracellular glutamate levels (Konradsson et al., 2009; Wu et al., 2010). The experiments in this thesis further investigated the interaction between KYNA and prefrontal glutamate release, revealing that systemic administration of KYNA's bioprecursor kynurenine results in dose-dependent reductions in cortical glutamate levels in the PFC. Furthermore, systemic co-administration of the positive α7 nAChR modulator galantamine either prevented or attenuated this reduction of extracellular glutamate release. These data, as recorded by the MEA, yielded results that were qualitatively and quantitatively similar to those produced by conventional techniques like microdialysis, further validating this novel electrochemical detection system.

KYNA as an astrocyte-derived neuromodulator

KYNA is a metabolite of the kynurenine pathway of tryptophan degradation (Kiss et al., 2003; see Figure 1), and is formed by the KAT-catalyzed transamination of its bioprecursor kynurenine in astrocytes (Guidetti et al., 2007), then is subsequently released into the extracellular milieu (Turski et al., 1989; see Figure 3). Initially, KYNA was reported to be a broad-spectrum antagonist of excitatory amino acid (EAA) receptors with low (millimolar) potency (Foster et al., 1984; Swartz et al., 1990). Later it was discovered that KYNA also binds competitively to the $glycine_B$ site of the NMDA-R (Perkins and Stone, 1982), reducing receptor activity in the absence of endogenous glycine levels (IC_{50} = ~10 μM; Kessler et al., 1989). However, in the presence of physiological levels of glycine, KYNA is less likely to inhibit the NMDA-R (IC_{50} = ~230 μM; Hilmas et al., 2001). More recently, KYNA was discovered to non-competitively

antagonize α7 nAChRs in physiologically relevant (nanomolar) concentrations (IC_{50} = 13.9 ± 8.3 μM; Hilmas et al., 2001; Lopes et al., 2007). Thus, α7 nAChRs could be considered a selective target for endogenous KYNA *in vivo* (Stone, 2007). Due to the localization of release-promoting α7 nAChR on cortical glutamatergic and cholinergic nerve terminals (Gotti et al., 2006; Lambe et al., 2003; Dickinson et al., 2008; Duffy et al., 2009) as well as the proximity of cholinergic and glutamatergic terminals in the PFC (Marchi et al., 2002; Rousseau et al., 2005; Wang et al., 2006; Konradsson-Geuken et al., 2009), the current experiments utilized the systemic administration of kynurenine to study the α7 nAChR-mediated relationship between KYNA and glutamate in the PFC.

While KYNA cannot cross the blood-brain barrier easily due to its polar chemical structure, kynurenine promptly enters the brain following peripheral administration (Gal and Sherman, 1978) utilizing an amino acid carrier that transports abundant, neutral amino acids such as tryptophan (a precursor to kynurenine; Fukui et al., 1991). Recent studies demonstrated that systemic administration of kynurenine dose-dependently raised KYNA levels in the PFC. Importantly, this increase in KYNA and the subsequent decrease in prefrontal glutamate levels (observed in the current experiments) was similar to the effect induced by the *local* perfusion kynurenine into the PFC (Wu et al., 2010). These parallel data demonstrate that increasing the concentration of KYNA elsewhere in the brain does not seem to interfere with the KYNA-mediated regulation of glutamate release in the PFC.

Regulation of prefrontal glutamate by KYNA: the role of α7 nAChRs

While it is clear that nicotine binding enhances glutamatergic transmission in cortex, both muscarinic and nicotinic ACh receptors (and various subtypes thereof) have been implicated in the nicotine-induced glutamate release observed in the PFC (Toth et al., 1993; Vidal and Changeux, 1993). The selective activation of α7 nAChRs by

choline, in particular, has been shown to elevate prefrontal glutamate levels (Konradsson-Geuken et al., 2009). Likewise, the selective inhibition of α7 nAChRs by the non-competitive antagonist KYNA via systemic administration of kynurenine (25 and 50 mg/kg, i.p.) results in reduced glutamate levels in the PFC (See Figures 6 and 7). Lastly, systemic co-administration of the positive α7 nAChR modulator galantamine (3 mg/kg, i.p.; both 5 min pre- and 3 hrs post-injection of kynurenine), which binds to an allosteric site similar to that bound by KYNA (Lopes et al., 2007), attenuates this kynurenine-mediated reduction in extracellular glutamate (See Figure 8). These observations suggest that endogenous KYNA *continuously* regulates extracellular glutamate levels in the PFC by acting at α7 nAChRs.

Functional and Clinical Implications

Dysregulations in glutamatergic and cholinergic transmission contribute to the pathophysiology underlying the cognitive deficits observed in schizophrenia (see Introduction). As such, these experiments, which further illuminate the bi-directional modulation of prefrontal glutamate release by KYNA, are of critical import in understanding the etiology and improving treatment strategies for patients' cognitive symptoms. As schizophrenic patients have abnormally elevated KYNA levels in the PFC (Schwarcz et al., 2001), it is consistent that they also exhibit deficits in several cognitive functions that are mediated by the PFC such as working memory, attention, and cognitive flexibility (Andraeson, 1995). Likewise, animals with experimentally-elevated KYNA levels also exhibit performance deficits in behavioral tasks requiring cognitive processes similar to those that are impaired in schizophrenia (Erhardt et al., 2004; Chess et al., 2007). Recent clinical studies with schizophrenic patients reported modest improvement in cognitive functioning upon treatment with partial α7 nAChR agonists (Biton et al., 2007), positive α7 nAChR modulators like galantamine (Thomsen et al.,

2010), or glutamatergic agonists (Coyle, 2006). Taken together, these data suggest that adjunctive treatment strategies that are designed to attenuate the production of KYNA in the brain or counter its effects on α7 nAChRs may be efficacious in the treatment of cognitive deficits in schizophrenia (Wonodi and Schwarcz, 2010).

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FIGURES AND FIGURE LEGENDS

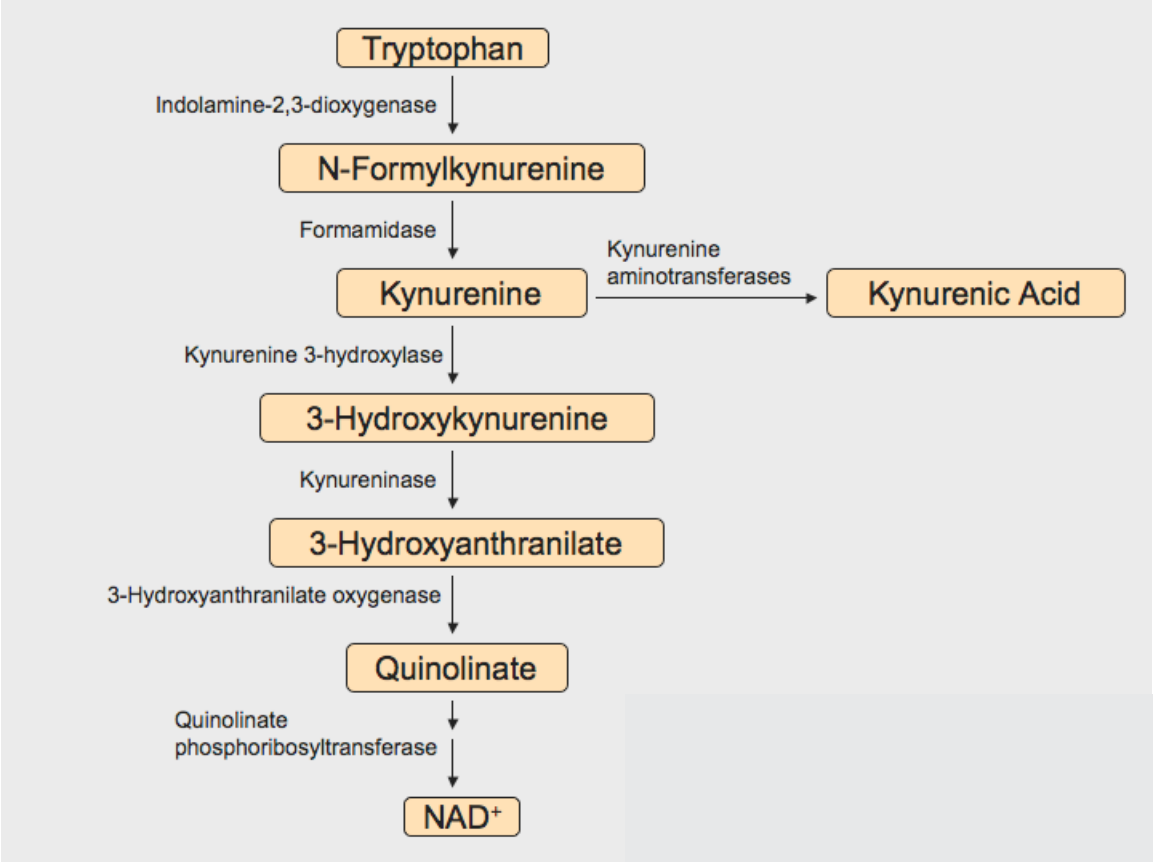

Figure 1. The kynurenine pathway of tryptophan degradation (Nilsson, 2005; Erhardt *et al.*, 2007).

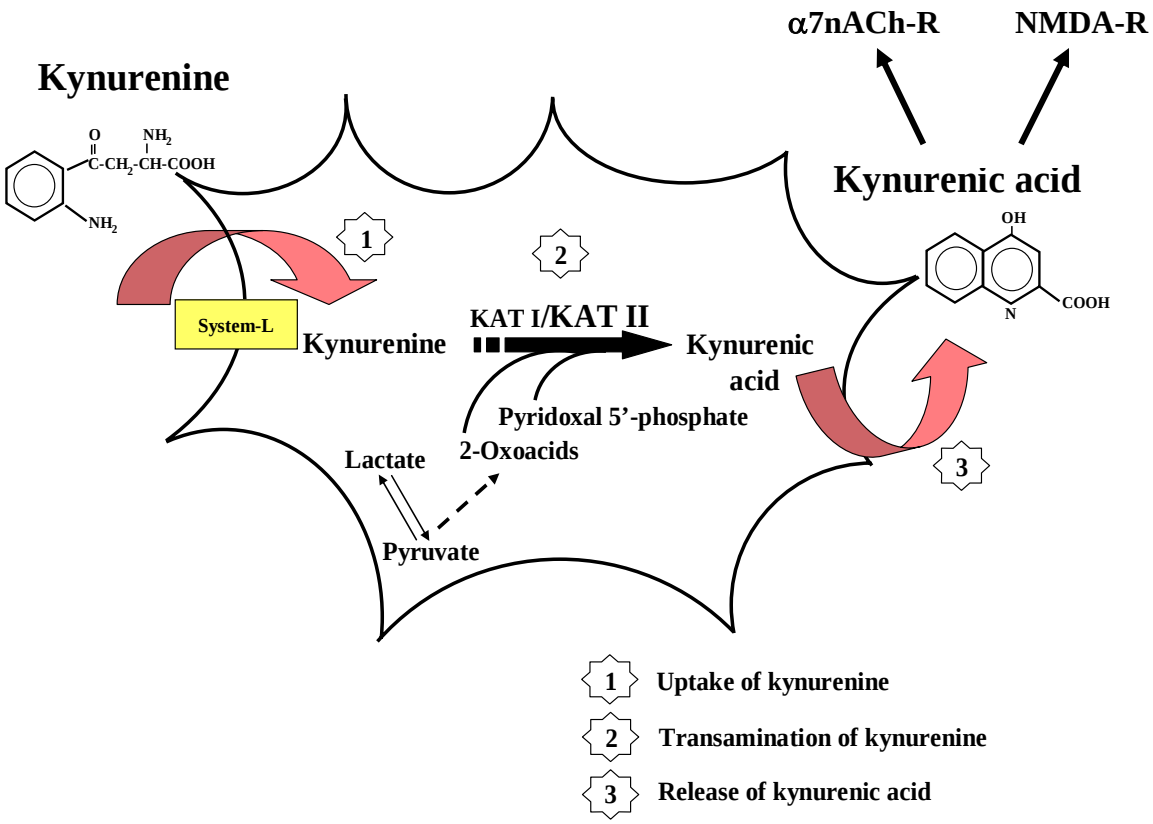

Figure 2. The transamination of kynurenine to kynurenic acid in astrocytes. The affinity of the α7 nAChR for KYNA (IC_{50} of α7 nAChR:13.9 ± 8.3 μM; Lopes et al., 2007) is similar to that of the $glycine_B$ site of the NMDA-R in the absence of glycine (IC_{50} of NMDA-R in the absence of glycine: ~10 μM; Kessler et al., 1989). In physiological conditions, however, KYNA is unlikely to inhibit the NMDA-R (IC_{50} of NMDA-R in the presence of glycine: ~230 μM; Hilmas et al., 2001), whereas KYNA will negatively modulate α7 nAChR at nanomolar concentrations (Hilmas et al., 2001). (Figure provided by Dr. Robert Schwarcz, University of Maryland)

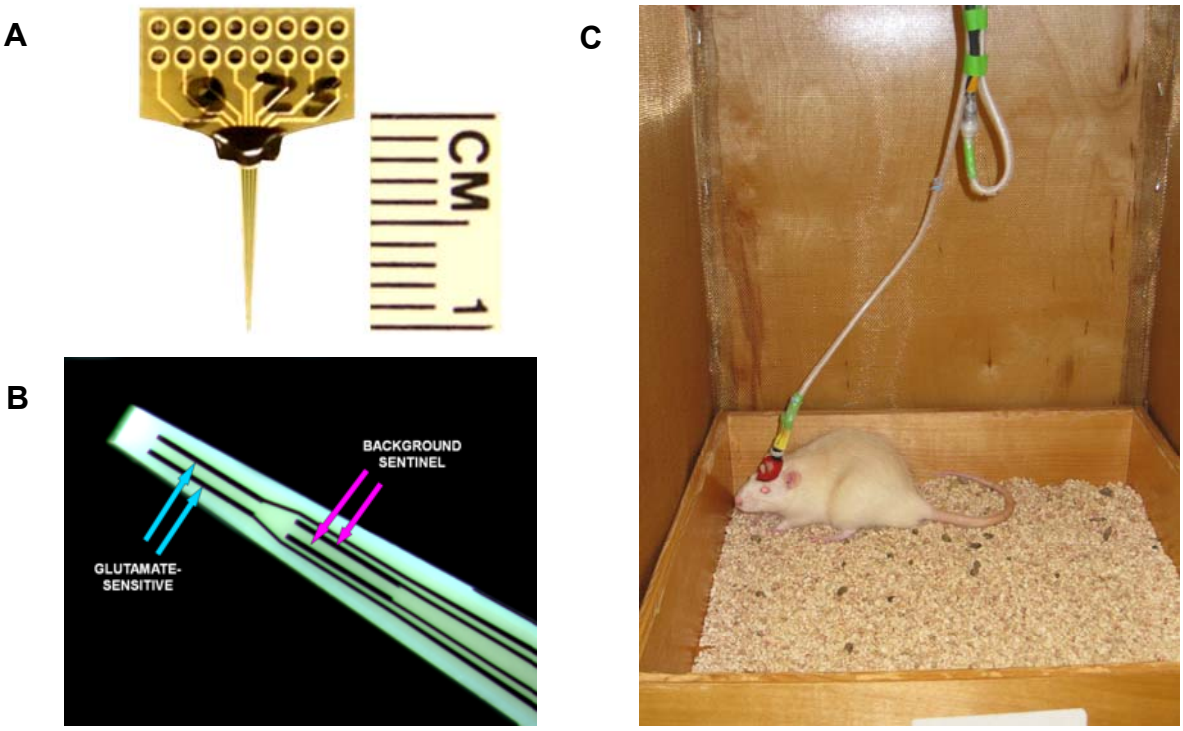

Figure 3. The glutamate-sensitive microelectrode array (MEA) and the enzyme scheme used in the detection of glutamate. **A:** Photograph of the MEA with ceramic paddle and recording channels. **B:** High magnification of the tip of the MEA illustrating the two pairs of recording sites, one pair sensitive to glutamate and the remaining pair as a sentinel for background (non-glutamate derived) signals. **C:** Recording of freely moving animals.

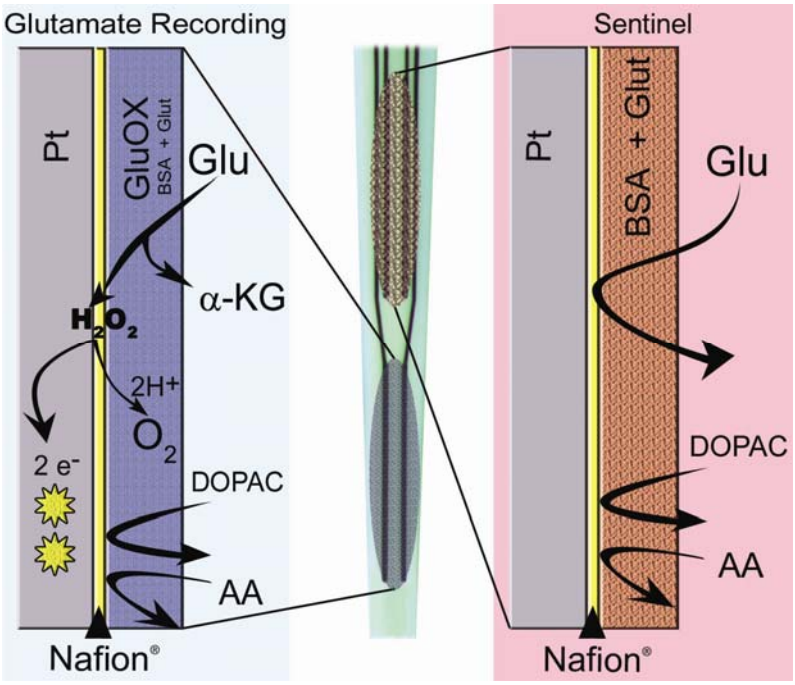

Figure 4. Enzyme detection scheme. **Glutamate Recording channels:** Glutamate is oxidized by glutamate oxidase (GluOx), generating α-ketoglutarate and H_2O_2. Because the MEA is maintained at a constant potential of +0.7 V, the H_2O_2 reporting molecule is oxidized, yielding two electrons. The resulting current is then amplified and recorded by a FAST-16 m*kI* recording system. **Sentinel channels:** Extracellular glutamate reaches the Pt surface but in the absence of Glu Ox no oxidation current is generated. Any current detected is due to endogenous electroactive molecules other than glutamate.

***Nafion®** is a sulfonated tetrafluoroethylene based fluoropolymer-copolymer discovered in the late 1960s. Its membrane exhibits an ionic gradient permeable to cations but not anions or electrons. Instead of Nafion®, however, these experiments utilized the exclusion barrier ***m*-PD** (see Methods). *Following* enzyme coating, *m*-PD (5.0 mM) was electropolymerized onto all sites using cyclic voltammetry in order to reduce access of potential electroactive interferents, including AA and catecholamines, to the Pt recording sites (cf. Figure 5). (Figure adapted from Konradsson-Geuken, 2009)

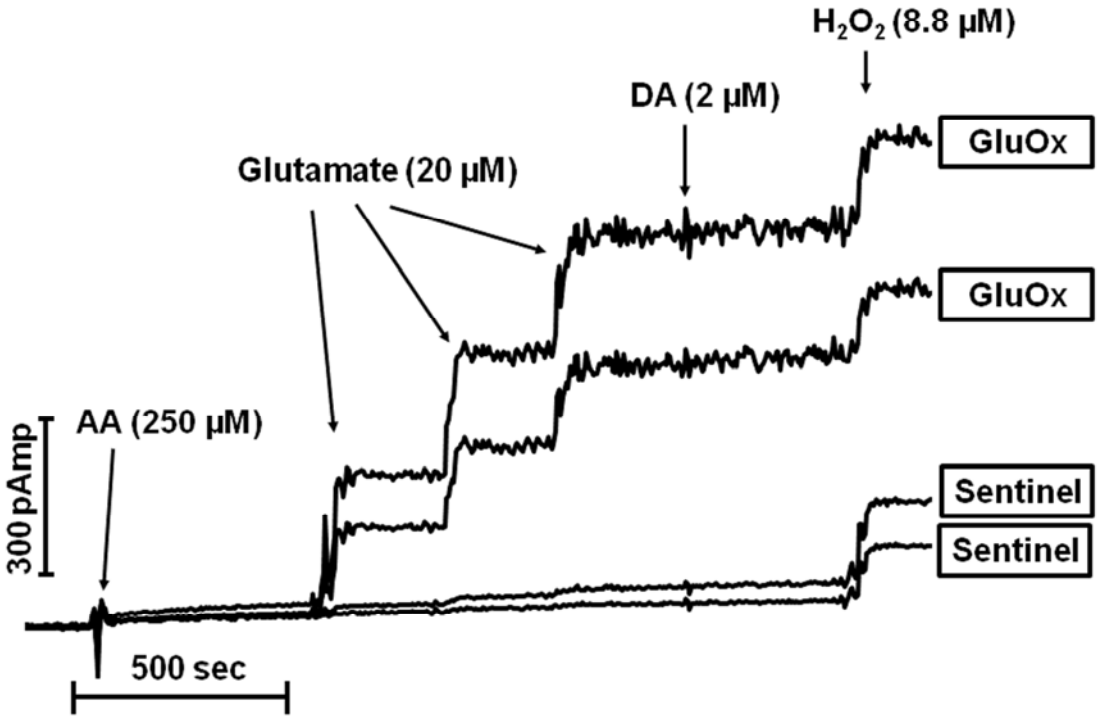

Figure 5. Representative *in vitro* calibration of the glutamate-sensitive MEA immediately prior to implantation into the PFC. Top two tracings: glutamate-sensitive (GluOx) recording channels. Bottom two tracings: sentinel background channels. Arrows indicate addition of various substances to the calibration beaker. Current (pAmp) is depicted along the vertical axis, and time (sec) along the horizontal axis. Successive additions of glutamate (raising concentration by 20 µM/aliquot) produce a linear increase in the glutamate signal. No glutamate-related changes in current are detected on the two sentinel channels. Note equivalent sensitivities to the reporter molecule H_2O_2 on all four channels. *m*-PD coating blocked access of negatively charged *in vivo* interferents such as ascorbic acid (AA) or dopamine (DA) (see Methods).

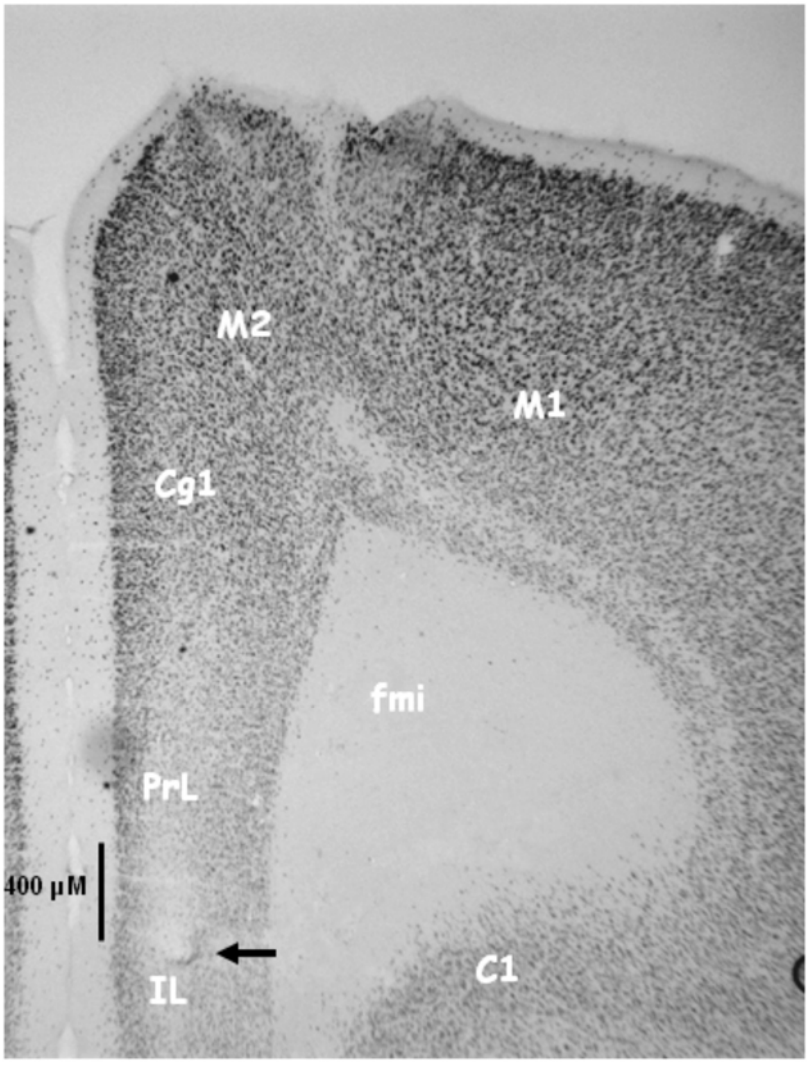

Figure 6. Photomicrograph illustrating a representative placement of MEA within prefrontal cortex (PFC). (A) frontal section depicting the position of a microelectrode in the ventral prelimbic region of PFC. The termination of the Pt tip of the MEA is depicted by the arrow above the infra-limbic (IL) cortex. Note the modest tissue disruption produced by the implanted MEA. This is consistent with previous reports from our group (Rutherford et al., 2007; figure adapted from Konradsson et al., 2009).

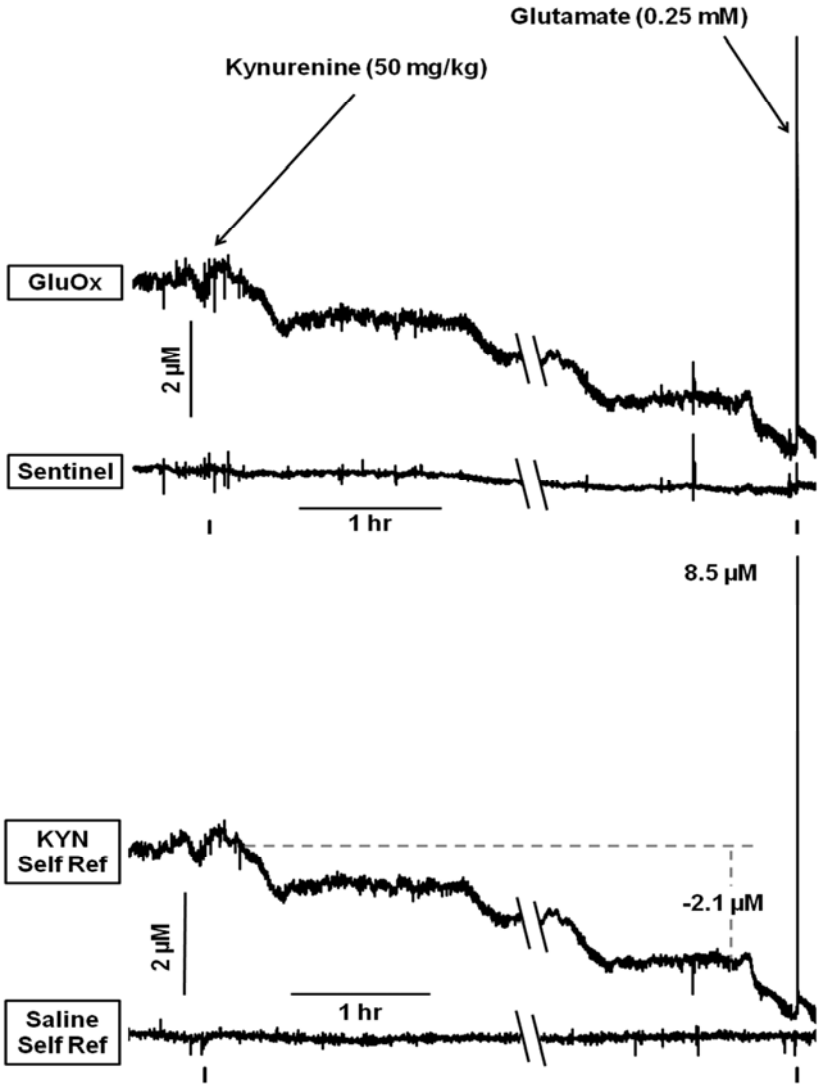

Figure 7. Representative MEA tracings from a rat receiving kynurenine (KYN; 50 mg/kg, i.p.). Top two tracings: MEA signal from the glutamate-sensitive site (GluOx) and its adjacent background sentinel. The third trace from the top reflects the self-referenced signal (Self Ref) of the GluOx channel against the sentinel background. Deflections along the vertical axis reflect changes in concentration (µM), and the horizontal axis depicts time (hr). The total recording session extended for 6 hrs after kynurenine administration (note 3 hr break in time axis). Kynurenine produces a steady decline in the glutamate signal, reaching a maximum decrease of 2.1 µM by the end of

the recording session (see Table 1 for group data). A control local infusion of glutamate (0.25 mM) rapidly elicits the characteristic phasic glutamate signal (8.5 μM increase), demonstrating that the MEA remained sensitive to changes in glutamate levels at the nadir of the kynurenine effect. Bottom tracing: self-referenced signal from an animal that received a control saline injection (0.9%, i.p.), demonstrating stability of the basal glutamate signal.

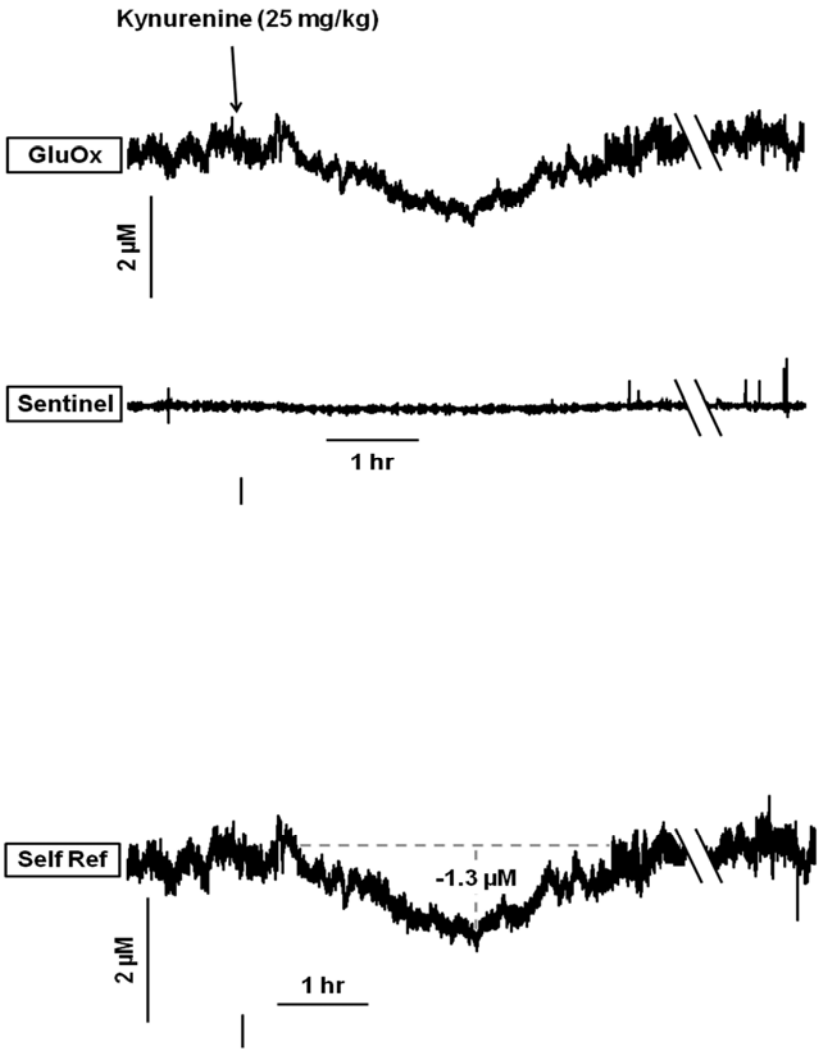

Figure 8. Representative MEA tracings from a rat receiving an injection of kynurenine (25 mg/kg, i.p.). The self-referenced glutamate signal (bottom tracing; Self Ref) reveals that 25 mg/kg kynurenine produces a smaller maximal decrease in glutamate (1.3 µM) than 50 mg/kg of kynurenine (cf. Figure 7). Note the 3 hr break in the time axis. Glutamate levels return to basal values within 3 ½ hrs (see Table 1 for group data).

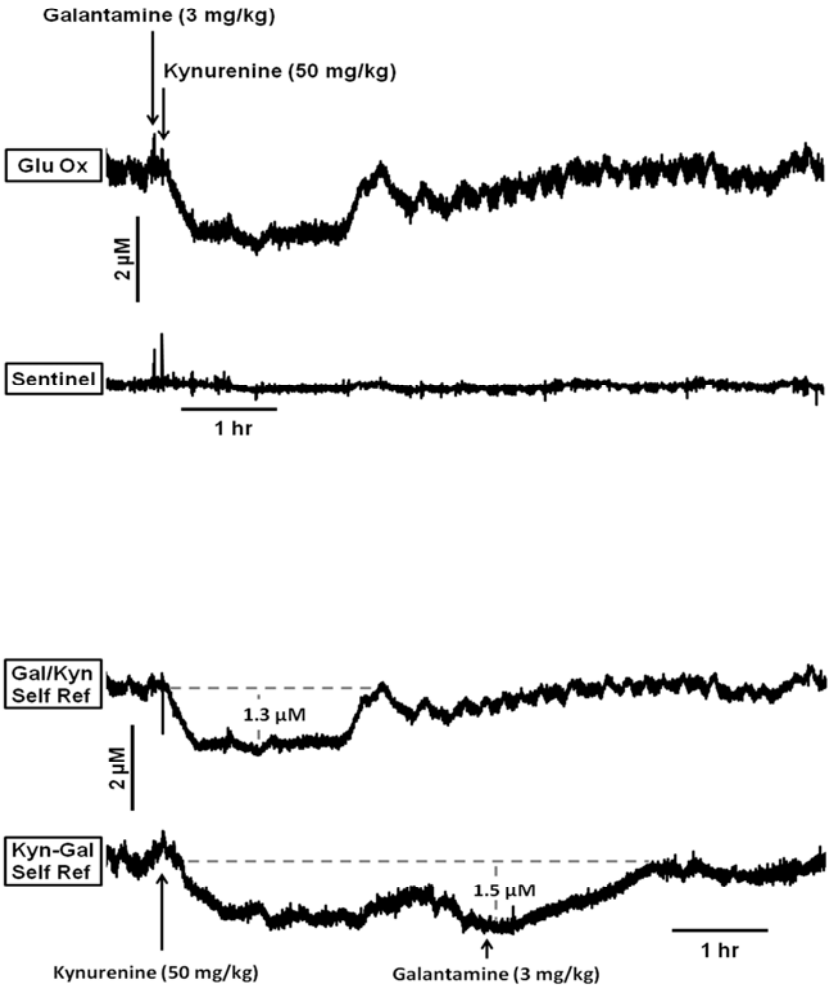

Figure 9. Representative MEA tracings from a rat receiving an injection of galantamine (3 mg/kg, i.p.), followed, 5 min later, by kynurenine (50 mg/kg, i.p.). Top two tracings: MEA signal from the glutamate-sensitive site (GluOx) and its adjacent background sentinel. The third tracing, illustrating the self-referenced (Self Ref) glutamate signal, reveals that galantamine attenuates the amplitude of the decline and the time course to values similar to those seen following 25 mg/kg kynurenine (cf. Figure 8 and Table 1). Bottom tracing: self-referenced record from an animal that first received kynurenine (50 mg/kg, arrow) and then, 3 hrs later at the nadir of glutamate levels, galantamine (3 mg/kg, arrow). The glutamate signal gradually returns to basal values following delayed galantamine administration, suggesting that the kynurenine-induced attenuation of glutamate release is *consistently* mediated by α7 nAChRs.

GLUTAMATE LEVELS FOLLOWING SYSTEMIC INJECTIONS:

GROUP MICROELECTRODE DATA

MEASURE	KYNURENINE (25 mg/kg)	KYNURENINE (50 mg/kg)	GALANTAMINE (3 mg/kg) + KYNURENINE (50 mg/kg)
BASAL GLUTAMATE (μM)	6.0 ± 1.3	6.2 ± 1.4	6.5 ± 1.6
POST-INJECTION GLUTAMATE (% CHANGE FROM BASAL)	-10.6 ± 1.1[a]	-30.8 ± 5.0	-10.8 ± 2.3[a]
TIME TO EFFECT (min)	16.2 ± 4.9	5.4 ± 3.6	16.3 ± 8.9
TIME TO MAX EFFECT (min)	111.2 ± 23.3[a]	314.0 ± 20.0	77.8 ± 20.2[a]
DURATION OF EFFECT (min)	210.3 ± 21.6[a]	> 6 hr	146.6 ± 13.4[a,b]

Table 1. All pharmacological treatments were administered via intraperitoneal (i.p.) injections. Values are means ± S.E.M. with n = 5 rats/treatment group. [a] = significantly different from kynurenine (50 mg/kg); [b] = significantly different from kynurenine (25 mg/kg).